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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE, VIII**

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In the preceding paper[1] code triplets containing no uracil were assigned to several amino acids as a result of studies with poly A, poly AC, and poly AG. Results with poly ACG, to be reported in this paper, have led to the assignment of 1A1C1G triplets to the amino acids alanine, aspartic acid, and serine. Moreover, poly C has now been found to stimulate the incorporation of proline into acid-insoluble products by the *E. coli* system, leading presumably to the formation of polyproline. Thus, CCC is a code letter for proline. Previous negative results[2] can be explained by (*a*) low affinity of poly C for the ribosomes, and (*b*) partial solubility of polyproline in 5 per cent trichloroacetic acid. In the present experiments poly C was used at a concentration of 200 μg/0.25 ml of reaction mixture and 20 per cent (final concentration) trichloroacetic acid was employed as the precipitating agent. C-rich polymers could therefore be used in the search for additional code letters. Experiments with poly CI, where inosinic acid takes the place of guanylic acid,[3]

have yielded three new 2C1G code letters for the amino acids alanine, arginine, and threonine, and one 1C2G letter for glycine.

Results of experiments with polynucleotides containing analogs of the naturally occurring bases will also be reported in this paper.

Preparations and Methods.—As in the previous paper,[1] the nucleoside 5'-diphosphates used in the preparation of the various polynucleotides were purified by ion-exchange chromatography. Poly ACG (6:1:1), poly ACG (4:1:1), poly C, and poly CI (5:1) were prepared with *Azotobacter* polynucleotide phosphorylase as previously described.[4] The sedimentation coefficients of poly ACG (4:1:1) and poly ACG (6:1:1) were 1.6 and 1.7 *S*, respectively. We are indebted to Dr. J. Y. Chen for these determinations. Base analyses of the copolymers used in this work have not yet been performed.

Polynucleotides containing analogs of the naturally occurring bases were prepared with *Azotobacter* polynucleotide phosphorylase from the corresponding nucleoside 5'-diphosphates. 5-fluorouridine 5'-diphosphate was, as previously stated,[4] a gift of Dr. Charles Heidelberger. Synthetic N(3)-methyluridine 5'-diphosphate was kindly supplied by Dr. D. Shugar, Polish Academy of Sciences, Warsaw. Xanthosine 5'-diphosphate was prepared by deamination of commercial guanosine 5'-diphosphate with nitrous acid.

In all experiments with poly C and poly CI (5:1), 200 μg of the former and 100 μg of the latter polymer were used per 0.25 ml of reaction mixture. After incubation, 1.0 mg of carrier polyproline in 0.5 ml of water was added to each sample. This was followed by the addition of 5 ml of 20 per cent trichloroacetic acid. The samples were centrifuged and the precipitates resuspended in 10 ml of 20 per cent trichloroacetic acid, kept at 95° for 15 min and then cooled to room temperature. The precipitates were plated on Whatman No. 1 filter paper disks, washed with absolute acetone, dried, and their radioactivity was measured in a windowless gas-flow counter. The incubations with poly ACG (30 μg/0.25 ml of reaction mixture) were as described in the preceding paper[1] with use of trichloroacetic-tungstic acid as the precipitating reagent.

Results.—Effect of poly C on proline incorporation: Following the finding that poly A stimulated the incorporation of lysine,[1] it appeared desirable to reinvestigate the problem of whether or not poly C stimulated the incorporation of proline. It may be remembered that poly C had been reported to be active[5] for proline incorporation although later this activity appeared to be explainable by the presence of some uridylic acid residues in the poly C preparations used.[6] In our experience[2, 4] stimulation of proline incorporation by poly C was very small while copolymers containing both U and C were much more effective. Reinvestigation of the problem was made even more desirable by indications that poly C, in contrast to poly U, had low affinity for the ribosomes. Thus, while poly U effectively competed with poly UC and markedly decreased stimulation of serine incorporation by the latter polymer, poly C decreased this incorporation to a very slight extent. Poly C also decreased very slightly, if at all, the poly U-promoted incorporation of phenylalanine. In these experiments all polymers were used at a concentration of 160 μg/ml.

In view of the above, it was possible that, in previous experiments, poly C might have been essentially inactive not because of intrinsic meaninglessness of CCC triplets, but because of the low affinity of the polymer for the ribosomes. This view was substantiated by the experiment of Table 1 showing a marked polymer concentration dependence for proline incorporation in the presence of poly C. At high concentrations (800 μg/ml) the incorporation of proline was effectively promoted by this polymer. In this experiment 20 per cent trichloroacetic acid was used as the precipitating agent as it was found that only about half of the proline counts were recovered in acid-insoluble products when 5 per cent trichloroacetic

TABLE 1

PROLINE INCORPORATION IN *E. coli* SYSTEM AS A FUNCTION OF THE CONCENTRATION OF POLY C

Poly C (μg/ml)	Proline incorporation*
0	0.01
100	0.03
200	0.14
400	0.37
800	0.51

* mμmoles/mg ribosomal protein. Incubation, 60 min at 37°.

acid was used as it had been in previous work. Moreover, as already mentioned, cold polyproline was added as a carrier for precipitation. These results show that previous negative results with poly C were due mainly to low affinity for the ribosomes and, to a lesser degree, to partial solubility of polyproline in 5 per cent trichloroacetic acid. A CCC code letter can therefore be definitely assigned to proline.

Additional code triplet assignments: Table 2 lists the effects of poly ACG (4:1:1), poly ACG (6:1:1), poly C, and poly CI (5:1) on the incorporation of each of 20 amino acids. The trichloroacetic-tungstic procedure[1] was used for the experiments of columns 2, 3, and 4, and the 20 per cent trichloroacetic acid procedure, described in this paper, for those of columns 5, 6, and 7.

The basis of the code triplet assignments, listed in Table 3, in the case of the ACG copolymers is as explained previously[1] for A-rich copolymers. In the case of poly CI (5:1) the stimulation of the incorporation of a given amino acid by this polymer

TABLE 2

AMINO ACID INCORPORATION IN *E. coli* SYSTEM WITH VARIOUS POLYNUCLEOTIDES*

	Polynucleotide†					
Amino acid‡	None	ACG (4:1:1) 120 μg/ml	ACG (6:1:1) 120 μg/ml	None	C 800 μg/ml	CI (5:1) 400 μg/ml
Alanine (7.5)	60	181	126	24	32	132
Arginine (5.0)	40	590	390	15	12	100
Asparagine (1.32)	26	380	415	38	30	22
Aspartic acid (5.0)	19	97	78	18	12	12
Cysteine (7.0)	47	63	50	37	41	37
Glutamic acid (5.0)	27	458	500	14	12	11
Glutamine (2.0)	42	662	678	20	9	9
Glycine (7.8)	52	121	82	13	15	36
Histidine (4.0)	59	320	379	23	13	13
Isoleucine (5.5)	33	21	19	12	12	12
Leucine (6.0)	24	16	16	15	9	8
Lysine (1.0)	40	2100	2960	14	14	14
Methionine (4.75)	160	101	97	34	29	25
Phenylalanine (6.8)	23	24	24	15	13	16
Proline (10.0)	8	190	90	5	725	447
Serine (4.0)	34	190	137	22	22	22
Threonine (5.0)	42	536	610	20	24	93
Tryptophan (3.74)	109	108	...	76	76	79
Tyrosine (6.5)	21	10	10	7	6	7
Valine (5.0)	14	13	12	19	14	11

* $\mu\mu$moles/mg ribosomal protein. Different precipitation procedures (see *Methods*) were used in the experiments of columns 2, 3, and 4, and columns 5, 6, and 7 of the table. Values for new code triplet assignments are enclosed in rectangles; all other polynucleotide-stimulated amino acid incorporations are underlined.

† Base ratios given are the ratios of ribonucleoside diphosphates used in the preparation of the polymers.

‡ Values in parentheses give specific radioactivities in μc/μmole.

TABLE 3

ADDITIONAL CODE TRIPLET ASSIGNMENTS*

	Polynucleotide						
	ACG (4:1:1)		ACG (6:1:1)		CI (5:1)		
	Found	Calculated	Found	Calculated	Found	Calculated	Code triplets†
Alanine	5.9	6.3 + 1.6 = 7.9	2.3	2.8 + 0.5 = 3.3	22.5	20.0	*1A1C1G, 2C1G*
Arginine	26.7	25.0 + 1.6 = 26.6	12.0	16.7 + 0.5 = 17.2	19.2	20.0	2A1G, *2C1G*
Asparagine	17.2	25.0	13.5	16.7	0	...	2A1G
Aspartic acid	3.8	6.3	2.0	2.8	0	...	*1A1C1G*
Glutamic acid	21.0	25.0	16.2	16.7	0	...	2A1G
Glutamine	30.0	25.0 + 6.3 = 31.3	21.8	16.7 + 2.8 = 19.5	0	...	2A1C, 1A2G
Glycine	3.4	6.3 + 1.6 = 7.9	1.0	2.8 + 0.5 = 3.3	5.2	4.0	1A2G, *1C2G*
Histidine	12.7	6.3	11.0	2.8	0	...	1A2C
Lysine‡	100.0	...	100.0	...	0	...	AAA
Proline‡	8.8	6.3 + 1.6 = 7.9	2.8	2.8 + 0.5 = 3.3	100	...	1A2C, *CCC*
Serine	7.6	6.3	3.5	2.8	0	...	*1A1C1G*
Threonine	24.0	25.0 + 1.6 = 26.6	19.5	16.7 + 0.5 = 17.2	16.5	20.0	2A1C, *2C1G*

* The polynucleotide-stimulated incorporation of each amino acid is given as percentage of lysine incorporation by ACG copolymers and of proline incorporation by CI copolymer (Found columns). This is compared with the percentage frequency of certain triplets to frequency of AAA triplets in the ACG copolymers and to frequency of CCC triplets in the CI copolymer (Calculated columns). Agreement or proximity of the two values is the basis for the code triplet assignments in the last column of the table.

† New code triplets are in italics.

‡ Incorporation taken as 100%.

relative to that of proline (taken as 100 per cent) was matched to the calculated per cent frequency of a given triplet relative to that of the CCC triplet in this polynucleotide. All of the matched values, on which the assignment of new additional code triplets (in italics in the table) to alanine (1A1C1G, 2C1G), arginine (2C1G), aspartic acid (1A1C1G), glycine (1C2G), serine (1A1C1G), and threonine (2C1G) is based, agree fairly closely. In the experiments with poly CI, I is considered to be equivalent to G since, in previous experiments,[3] poly UI has been shown to be equivalent to poly UG. Hence, the assignment of C and G-containing code letters based on experiments with poly CI is justified.

Experiments with polynucleotides containing analogs of naturally occurring bases: At the concentrations tried, poly N-methyluridylic acid (poly MeU) and polyfluorouridylic acid (poly FU) did not significantly stimulate incorporation of phenylalanine by the *E. coli* system (Table 4). The insignificant activity of the latter polymer has been previously noted.[4] As also shown in Table 4, the UMeU (9:1) copolymer had one-tenth the activity of poly U, but UFU copolymers were quite active. There is no obvious reason why poly FU (a polymer of 5-fluorouridylic acid) should be inactive unless this is due to a lack of affinity for the ribosomes. On the other hand, in poly MeU (a polymer of N(3)methyluridylic acid) a hydrogen bonding site, the number 3 ring nitrogen, is blocked by a methyl group, and this polymer does not form a twin-stranded complex with poly A.[7] This would interfere with attachment of the AAA adaptor[8] triplet in phenylalanine transfer RNA to the polynucleotide template.

From the results with poly UMeU and poly UFU (Table 4) it would appear that 5-fluorouracil which can pair with adenine through hydrogen bonding, is as effec-

TABLE 4

PHENYLALANINE INCORPORATION IN *E. coli* SYSTEM WITH POLYNUCLEOTIDES CONTAINING XANTHYLIC ACID AND BASE ANALOGS OF URIDYLIC ACID

Polynucleotide (160 μg/ml)	Phenylalanine incorporation*
None	0.1
Poly U	4.9
Poly N-methyl U (MeU)	0
Poly UMeU (9.1)†	0.5
Poly fluoro U (FU)	0.05
Poly UFU (5:1)‡	4.1
Poly UFU (6:4)†	2.2§
Polyuridylic-xanthylic acid (UX) (9:1)†	0.6

* mμmoles/mg ribosomal protein. Incubation, 60 min at 37°. Other conditions as previously described.[4]
† Actual base ratio of polynucleotide.
‡ Ratio of nucleoside diphosphates used in preparation of polymer.
§ In another experiment the value was 4.5. While poly U saturated the system at a concentration of 80 μg/ml, poly UFU (6:4) did not reach saturation at 400 μg/ml.

tive in coding as uracil when the FU residues are copolymerized with U residues, whereas N(3)methyluracil which, through lack of a hydrogen bonding site cannot pair with adenine, is not effective. The activity of the UFU copolymer and the inactivity of the FU homopolymer pose an interesting problem which cannot be explained without further experimental work. The different behavior might be related to ribosomal binding in the former case and lack of binding in the latter.

Comparison of the effects of poly UMeU (9:1) and poly UX (9:1) indicates that both the N-methyluracil and the xanthine residues are inactive in coding. Incorporation of phenylalanine into products insoluble in 5 per cent trichloroacetic acid was reduced in either case to about 10 per cent of the phenylalanine value with poly U (Table 4). These copolymers, because of the short uninterrupted U sequences (average, 10 residues) between meaningless N-MeU or X residues, might give rise to very short, mostly acid-soluble polyphenylalanine chains. The inactivity of xanthine in coding has been previously pointed out.[3]

It may be added that the UX (9:1) copolymer did not promote the incorporation of leucine, serine, tyrosine, or valine, suggesting that X cannot replace A, C, or G. The same was true of poly UFU (6:4). However, due to the limited sensitivity of our assay system, a very small incorporation of these amino acids would have escaped detection.

Discussion and Conclusions.—The results of previous work[1, 9] and the work reported in this paper bring to 41, out of 64 triplets in RNA, the number of triplets in the amino acid code, and the list is probably still incomplete. It may be mentioned that recent experiments with poly UCG (6:1:1) failed again to substantiate the prediction[10] that 1U1C1G is a code triplet for glutamine while, on the other hand, incorporation of this amino acid was promoted by poly AC and poly AG.[1] Hence, 1U1C1G has been excluded from the list as a glutamine triplet. Also, previous results suggesting 1U2C as a code triplet for threonine could not be confirmed, and this has been removed from the list as a threonine triplet.

A summary of the code triplet assignments to date is shown in Table 5. The U-containing triplets have been arranged in the sequences proposed by Jukes.[11] When possible, the sequence of non-U triplets was fitted to that of U-triplets as if triplets for a given amino acid were derived from each other through a single base replacement, e.g., GAG and GCG for an assumed GUG sequence of the U-containing glycine triplet. When this was not possible, non-U sequences for an amino acid

TABLE 5

AMINO ACID CODE TRIPLETS

Amino acid	U-triplets*	Non-U triplets	Shared doublets
Ala	CUG	CAG, CCG	C●G
Arg	GUC	GAA, GCC	G●C
AspN	UAA, CUA	CAA	●AA, C●A
Asp	GUA	GCA	G●A
Cys	GUU	...	...
Glu	AUG	AAG	A●G
GluN	...	AGG, AAC	...
Gly	GUG	GAG, GCG	G●G
His	AUC	ACC	A●C
Ile	UUA, AAU†	...	...
Leu	UAU, UUC, UGU	...	U●U
Lys	AUA	AAA	A●A
Met	UGA	...	...
Phe	UUU	...	...
Pro	CUC	CCC, CAC	C●C
Ser	CUU	ACG	...
Thr	UCA	ACA, CGC	●CA
Try	UGG	...	...
Tyr	AUU	...	...
Val	UUG	...	...

* Sequences from T. H. Jukes.[11]
† Not in Jukes' list.

were written to avoid duplication with sequences of the same base composition for another amino acid. All sequences are of course arbitrary, except for GUU and AUU for cysteine and tyrosine[12] since, with the possibility of extensive degeneracy of the code, sequence assignments based on amino acid replacement data cannot be made with any degree of certainty. It may be noted that in many cases (Table 5, column 4) a doublet is shared, with the same relative position of its bases, by two or three triplets of the same amino acid. It remains to be seen whether, in these cases, one or several transfer RNAs are involved in the read-out process. Weissblum *et al.*[13] have reported on two leucine trans-RNAs corresponding to the 2U1G and 2U1C code letters for this amino acid.

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A MULTIPLE RIBOSOMAL STRUCTURE IN PROTEIN SYNTHESIS

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It has been well established that the ribosomal particles are the site of protein synthesis, yet we have very little insight into the mechanism.[1] A great deal of attention has been directed toward the question of how the ribosomes contain the information necessary to effect the alignment of amino acids in a specific sequence. This problem has been resolved recently with the discovery of a rapidly metabolizing fraction of RNA, called messenger RNA, which has the ability to attach itself to the ribosomal particle and there to determine the sequence of amino acids.[2, 3] This view has been considerably reinforced by *in vitro* experiments in which naturally occurring RNA, as well as synthetic polyribonucleotides, have been shown to provide the information necessary to determine the sequence of amino acids in a polypeptide chain.[4, 5] Thus, the ribosome has a passive role in transmitting information; it can apparently polymerize a variety of proteins, depending upon the particular messenger RNA which is attached to it.

However, this state of affairs has puzzled many investigators for some time. The purely geometric aspects of the messenger RNA-ribosomal interaction leave several unresolved questions. For example, the polypeptide chains in the hemoglobin molecule each contain roughly 150 amino acids and, using a triplet code,[6] this implies a messenger of 450 nucleotides or a molecule 1,500 Å long if there is one nucleotide every 3.4 Å. How can this long polymer molecule transfer all of its sequence information to the ribosomal site at which the polypeptide chain is believed to grow? This becomes an even greater puzzle if one considers that much longer messenger RNA molecules have been found.[5] Indeed, the length is so great that it would almost be physically impossible for one ribosome 230 Å in diameter to interact with the entire messenger chain. However, Risebrough *et al.*[7] have shown that "heavy ribosomes" are seen in a sucrose gradient when labeled T2 messenger RNA is attached to *E. coli* ribosomes. More recently, several investigators[8–10] have reported that polyuridylic acid induces the formation of a rapidly sedimenting ribosomal peak when it is added *in vitro* to a cell-free bacterial extract. We have therefore been prompted to look for the possible existence of a larger multiple ribosomal structure *in vivo* which might provide insight into the detailed mechanism of protein synthesis.

In these experiments, we have used reticulocyte ribosomes because it is possible to break open the reticulocyte cell wall gently with a minimum of mechanical manipulation. This approach has been successful, and we have been able to demonstrate the existence of a multiple ribosomal structure held together by RNA. In